

A Functionally Significant Cross-talk between Androgen Receptor and ErbB2 Pathways in Estrogen Receptor Negative Breast Cancer¹

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Abstract

Recent studies have identified novel subgroups in ER-negative breast cancer based on the expression pattern of androgen receptor (AR). One subtype (molecular apocrine) has an over-expression of steroid-response genes and ErbB2. Using breast cancer cell lines with molecular apocrine features, we demonstrate a functional cross-talk between AR and ErbB2 pathways. We show that stimulation of AR and ErbB2 pathways leads to the cross-regulation of gene expression for *AR*, *ErbB2*, *FOXA1*, *XBP1*, *TFF3*, and *KLK3*. As opposed to the physiologic transient phosphorylation of extracellular signal-regulated kinase (ERK1/2) observed with the testosterone treatment, we demonstrate that the addition of ErbB2 inhibition leads to a persistent phosphorylation of ERK1/2, which negatively regulates the downstream signaling and cell growth. This suggests a mechanism for the cross-talk involving the ERK pathway. Moreover, testosterone stimulates the proliferation of molecular apocrine breast cell lines, and this effect can be reversed using antiandrogen flutamide and anti-ErbB2 AG825. Conversely, the growth stimulatory effect of heregulin can also be inhibited with flutamide, suggesting a cross-talk between the AR and ErbB2 pathways affecting cell proliferation. Importantly, there is a synergy with the combined use of flutamide and AG825 on cell proliferation and apoptosis, which indicates a therapeutic advantage in the combined blockage of AR and ErbB2 pathways.

Neoplasia (2008) 10, 542–548

Introduction

Estrogen receptor–negative (ER–) breast cancer constitutes approximately 30% of all cases and poses a significant management challenge due to the limited available therapeutic targets. In addition, the lack of functional ER in breast cancer has been associated with a more aggressive metastatic phenotype [1]. Therefore, identification of novel molecular targets in ER– breast cancer can result in more effective therapies. Using expression microarray analysis, Sorlie et al. [2,3] have classified breast cancer into luminal, basal, ErbB2-like, and normal breast-like subtypes. The basal, ErbB2-like, and normal breast-like subtypes all have a low expression of *ER*, and ErbB2-like subtype has an *ErbB2* amplification [2,3]. Furthermore, Sotiriou et al. [4] have demonstrated that ER– breast cancers form a separate gene expression cluster from ER+ cancers, indicating that they are two distinct molecular entities.

There is a growing body of evidence suggesting that ER– breast cancers are heterogeneous with different molecular subtypes. Farmer

et al. [5] have shown that androgen receptor (*AR*) expression level divides ER– breast tumors in two major gene expression clusters. These are ER–/AR– (basal) and ER–/AR+ (molecular apocrine) subtypes. There is a higher frequency of *ErbB2* over-expression (ErbB2+) in the molecular apocrine subtype [5]. Other studies have confirmed these findings and demonstrated that the molecular apocrine subtype has a gene signature similar to that of estrogen response [6,7]. Furthermore, a cell line model for the molecular apocrine subtype, MDA-MB-453,

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¹This study is funded by Fund & Friends of Addenbrookes Charity and Diamantina Institute for Cancer, Immunology and Metabolic Medicine.

Received 7 February 2008; Revised 29 March 2008; Accepted 29 March 2008

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DOI 10.1593/neo.08274

demonstrates an increased proliferation in response to the androgen treatment, which can be reversed using the antiandrogen agent flutamide [6]. The higher expression of *ErbB2* in the molecular apocrine subtype suggests a biologic significance for the coexpression of *ErbB2* and steroid-response genes in this subtype of breast cancer. Interestingly, in prostate cancer, the ErbB2/ErbB3 pathway regulates AR by stabilizing AR protein levels and optimizing binding of AR to promoter/enhancer regions of androgen-regulated genes [8]. Tyrosine phosphorylation of ErbB2 plays a key role in regulating androgen-mediated proliferation in prostate cancer, and ErbB2 signaling is required for the optimal transcriptional activity of AR by mechanisms such as tyrosine phosphorylation of the receptor [9–11]. Furthermore, simultaneous down-regulation of *AR* and *ErbB2* significantly impairs the survival of prostate cancer cells [12]. These findings suggest a cross-talk between AR and ErbB2 pathways in prostate cancer.

In this study, we investigate the cross-talk between AR and ErbB2 pathways in breast cancer molecular apocrine subtype and assess the potential therapeutic implications for our findings.

Materials and Methods

Statistical Analysis

To assess the coexpression of *ErbB2* and *AR* genes, we used the microarray data from a cohort of 135 breast tumors (ArrayExpress database accession number: E-UCON-1) [13]. This cohort included a total of 39 ER⁺ tumors. We normalized the gene-expression ratios for *ErbB2* and *AR* to the mean expression of each gene across the data.

All statistical analysis was done with the Statistical Package for the Social Sciences version 14.0 (SPSS Inc., Chicago, IL). Mann–Whitney U test was applied for the comparison of nonparametric data.

Cell Culture and Treatments

Breast cancer cell lines MDA-MB-453 and MDA-MB-361 were cultured in L15 medium (Invitrogen, Paisley, UK), 10% fetal bovine serum (FBS). Sum-190 and SK-BR-3 cell lines were cultured in Ham's F12, 5% FBS and McCoy's 5a, 10% FBS medium, respectively (Invitrogen). BT-474 and prostate cancer cell line LNCaP were grown in DMEM, 10% FBS and RPMI, 10% FBS, respectively (Invitrogen). For hormonal and inhibitor experiments, cells were incubated in phenol red-free DMEM (Invitrogen), 10% charcoal-dextran-treated serum (HyClone, Cramlington, UK).

Testosterone (Sigma, Dorset, UK) treatment was carried out at 100-nM and 1- μ M concentrations. Heregulin Beta-1 (Cambridge Biosciences, Cambridge, UK) was applied at 1-nM concentration. With the exception of apoptosis assays, flutamide (Sigma) treatment was done at 10 μ M and ErbB2 inhibitor AG825 (Calbiochem, Beeston, UK) was used at 5- μ M concentration. All cells were incubated in phenol red-free media/charcoal-dextran-treated serum 1 day before the hormonal/inhibitor treatments and medium was changed every 2 days.

Real-time Polymerase Chain Reaction and Western Blot Analysis

Total RNA extraction from cells lines was performed using TRI reagent (Sigma). Reverse transcription-polymerase chain reaction (RT-PCR) to assess the expression levels of *AR*, *ErbB2*, *FOXAI*,

XBPI, *TFF3*, and *KLK3* was performed using gene-specific *TaqMan* assays (Applied Biosystems, Foster City, CA). Housekeeping genes *HPRT1* and *RPLP0* were used as controls. Experimental procedures were performed following the manufacturer's instruction. Relative gene expressions were calculated as described before [14]. For the cell treatment experiments: Relative gene expression = gene expression in the treated group / average gene expression in the control group. All experiments were performed in four biologic replicates.

Western blots with mouse monoclonal anti-AR antibody (Abcam, Cambridge, UK) and rabbit polyclonal HER2 antibody (Cell Signaling, Arundel, Australia) were carried out at 1:100 and 1:1000 dilutions of primary antibody, respectively. Experiments were carried out using 5 μ g of each cell lysate. Ponceau staining (Sigma) was carried out as loading control.

Extracellular Signal-Regulated Kinase Activation Using ELISA

MDA-MB-453 cells were cultured in 96-well plates to 60% confluence and incubated in phenol red-free media/charcoal-dextran-treated serum 1 day before the treatments in the following groups: 1) no-treatment control, 2) testosterone (1 μ M), and 3) testosterone (1 μ M) + AG825 (5 μ M). Cells were preincubated in DMSO (groups 1 and 2) or AG825 (group 3) 1 day before the treatments. Enzyme-linked immunosorbent assay experiments were performed in two time points: a short incubation at 1 hour and a long incubation at 18 hours. The amounts of phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) and total-ERK1/2 proteins were measured using SuperArray CASE ERK1/2 ELISA kit (Tebu-bio, UK). All assays were performed in 16 biologic replicates at each time point. For each experimental group, the ratio of phospho-p65/total-p65 was obtained.

Proliferation Assay

MDA-MB-453 and Sum-190 breast cancer cells were grown in 96-well plates to 50% confluence. Cells were then treated in the following groups for 96 hours with a change of medium at 48 hours: 1) heregulin, 2) AG825, 3) heregulin + AG825, 4) testosterone, 5) flutamide, 6) testosterone + flutamide, 7) testosterone + AG825, 8) heregulin + flutamide, and 9) AG825 + flutamide at the concentrations described previously. Cells untreated and grown in the same conditions were used as controls. Proliferation was measured using Vybrant MTT Proliferation Assay Kit (Invitrogen). The percentage of change in proliferation was calculated relative to the untreated cells at the same time point. All experiments were performed in 16 biological replicates.

Apoptosis Assays

MDA-MB-453 and Sum-190 cells were grown in six-well plates on coverslips to 60% confluence. Cells were then incubated in serum-free medium overnight followed by treatment with flutamide and AG825 for 18 hours in the following groups: 1) no-treatment control, 2) flutamide at 10 and 40 μ M, 3) AG825 at 5 and 20 μ M, and 4) flutamide + AG825. Hoechst 33258 (Sigma) staining was performed as described before [15]. Annexin V-fluorescein isothiocyanate assay was performed using Annexin V-FITC fluorescence microscopy kit (BD Biosciences, Oxford, UK). All experiments were performed in four biological replicates.

Results and Discussion

AR and ErbB2 Coexpression in Molecular Apocrine Breast Cancer

To assess the over-expression of *AR* and *ErbB2* in ER– breast tumors, we analyzed the expression microarray data for these genes in a cohort of 39 ER– breast tumors [13]. We found that 28% of ER– tumors were both AR+ and ErbB2+ (Figure 1A). Furthermore, we noted the 50% of ER– tumors were AR+ and 73% of ErbB2+ cases were also AR+. These data show that most ER–/ErbB2+ tumors also over-express *AR*.

Cell Line Models for AR+/ErbB2+ in ER– Breast Cancer

To identify valid cell line models for the study of cross-talk between AR and ErbB2 pathways, we assessed the expression of *ErbB2* and *AR* in a number of ER– breast cancer cell lines with known *ErbB2* amplification. We tested ER– cell lines Sum-190, MDA-MB-453, and SK-BR-3 (Figure 1B). In addition, ER+/ErbB2+ breast cell lines BT-474 and MDA-MB-361 and prostate cancer cell line

LNCaP were used as ErbB2+ and AR+ controls, respectively. We found that Sum-190 and MDA-MB-453 cells had over-expression of both *ErbB2* and *AR* (Figure 1B). However, SK-BR-3 showed over-expression of only *ErbB2* and a low expression of *AR* (Figure 1B). This pattern of *AR* expression in MDA-MB-453 and SK-BR-3 cell lines has been previously reported [6]. We further confirmed the protein expression of AR and ErbB2 in Sum-190 and MDA-MB-453 cells using Western blot (Figure 1, C and D). These findings indicate that Sum-190 and MDA-MB-453 cell lines are AR+/ErbB2+ and provide a valid model for the study of cross-talk between AR and ErbB2 pathways.

Cross-regulation of Gene Expression between AR and ErbB2 Pathways

We first assessed the cross-regulation of selected genes between AR and ErbB2 pathways at the expression level. Expression was measured at baseline and after modulation of AR pathway with testosterone and ErbB2 pathway with heregulin and AG825. Testosterone treatment for 18 hours significantly increased the *ErbB2* expression

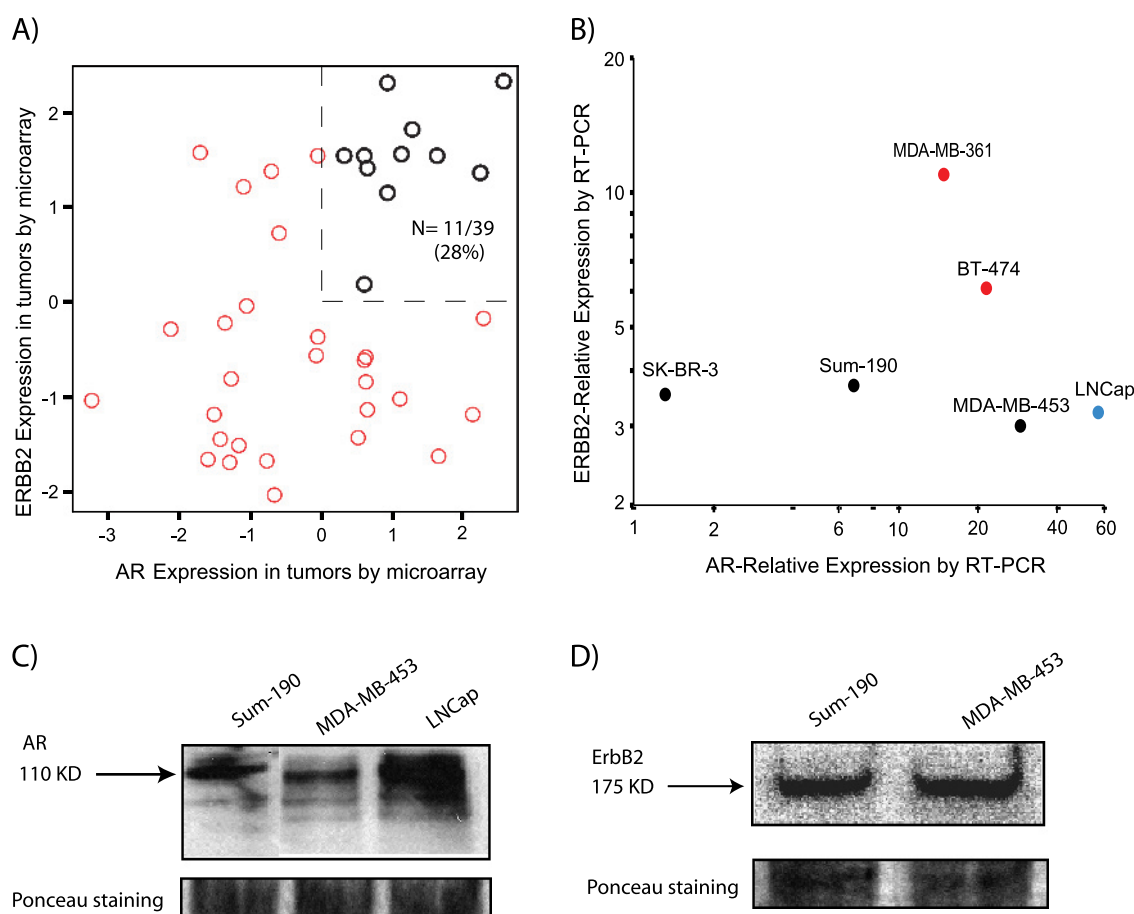


Figure 1. AR+/ErbB2+ subtype in estrogen receptor-negative breast cancer. (A) Scatter plot showing the correlation between *ErbB2* and *AR* expression using microarray data from a cohort of 39 ER– breast tumors. Cases with the over-expression of both *ErbB2* and *AR* are depicted in black. (B) Expression of *ErbB2* and *AR* measured using RT-PCR in Sum-190, MDA-MB-453, SK-BR-3, MDA-MB-361, and BT-474 breast cancer cell lines (black indicates ER–; red, ER+). LNCaP (blue) is a prostate cancer cell line. Expression is relative to that of normal breast RNA (Ambion, Warrington, UK). (C) Western blot to show AR protein expression in Sum-190, MDA-MB-453, and LNCaP cell lines. Ponceau staining is used as loading control. (D) Western blot to show ErbB2 protein expression in Sum-190 and MDA-MB-453 cell lines. Ponceau staining is used as loading control.

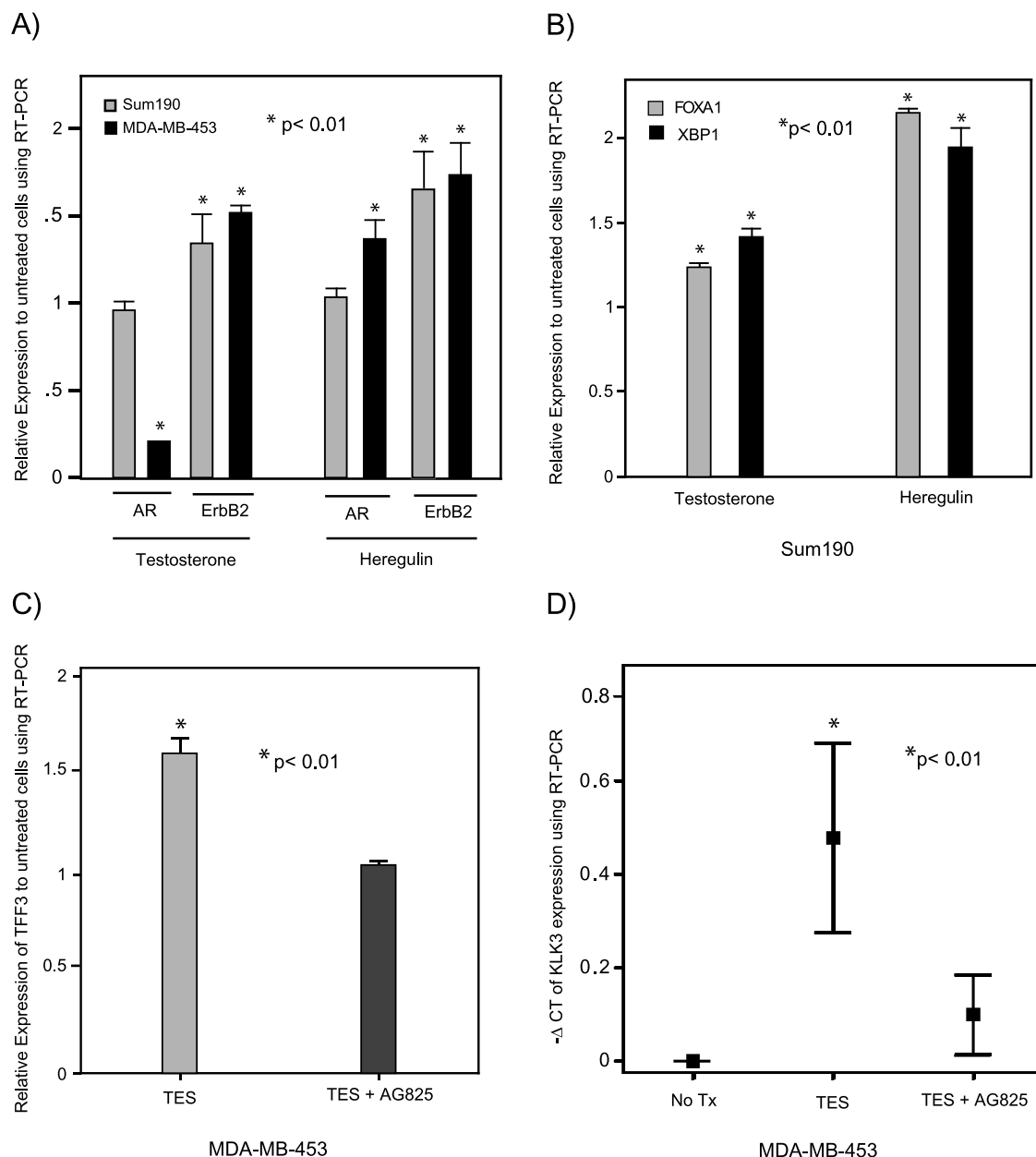


Figure 2. Cross-regulation of gene expression between AR and ErbB2 pathways. (A) Relative expression of *AR* and *ErbB2* using RT-PCR in MDA-MB-453 and Sum-190 cell lines after treatments with testosterone and heregulin. Expression is relative to that of untreated cells. (B) Relative expression of *FOXA1* and *XBP1* using RT-PCR in Sum-190 cell line after treatments with testosterone and heregulin. Expression is relative to that of untreated cells. (C) Relative expression of *TFF3* in the MDA-MB-453 cell line after treatments with testosterone (TES) and TES + AG825. (D) Expression change of *KLK3* shown as $-\Delta C_T$ in the MDA-MB-453 cell line after treatment with testosterone (TES) and TES + AG825. $-\Delta C_T = [-(C_T(KLK3) - C_T(\text{housekeeping genes}))]$ was applied because there is no baseline expression of *KLK3* in the MDA-MB-453 cell line. All data are shown for testosterone at a $1 \mu\text{M}$ concentration.

in MDA-MB-453 and Sum-190 cells ($P < .01$; Figure 2A). In addition, heregulin significantly increased the *ErbB2* expression in both cell lines and the expression of *AR* in MDA-MB-453 cells ($P < .01$; Figure 2A). We also studied the expression of steroid-response genes *FOXA1*, *XBP1*, and *TFF3* [6] and the AR signaling marker *KLK3* [gene for prostate-specific antigen (PSA) protein]. We observed a significant induction in the expression of *FOXA1* and *XBP1* with both testosterone and heregulin treatments in Sum-190 cells ($P < .01$; Figure 2B). Furthermore, in MDA-MB-453 cells, the expression of both *TFF3* and *KLK3* was significantly increased after

testosterone treatment for 18 hours ($P < .01$), and this was inhibited using AG825 (Figure 2, C and D). We confirmed that AG825 at $5 \mu\text{M}$ inhibits the phosphorylation of ErbB2 using ELISA (data not shown). It is notable that testosterone was used at two concentrations of 100 nM and $1 \mu\text{M}$, and we observed similar results with these concentrations. Moreover, testosterone did not stimulate *TFF3* and *KLK3* expression in Sum-190 cells. Interestingly, we observed a down-regulation of *AR* with the testosterone treatment in MDA-MB-453 cells (Figure 2A). This finding has been previously reported in other cancer cell lines such as LNCaP and is due to

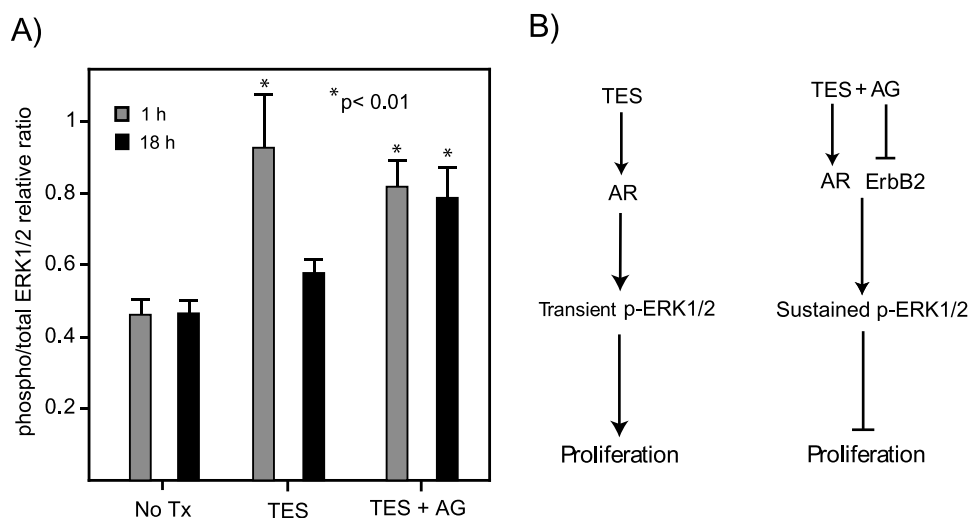


Figure 3. AR-ErbB2 cross-talk and ERK1/2 activation. (A) The relative ratio of phospho-ERK1/2/total-ERK1/2 using ELISA in MDA-MB-453 cell line. No Tx indicates no treatment; TES, testosterone at 1 μ M; AG, AG825 at 5 μ M. Cells were incubated for 1 hour (short incubation) and 18 hours (long incubation). * $P < .01$ is compared with the untreated cells using Mann-Whitney U test. (B) Schematic model for the cross-talk between AR and ErbB2 in molecular apocrine subtype of breast cancer. TES indicates testosterone; p-ERK1/2, phosphorylated ERK1/2; arrow, stimulatory effect; crossed line, inhibitory effect.

the transcriptional regulation of *AR* by testosterone [16]. These findings indicate that there is a cross-regulation in the expression of selected genes between AR and ErbB2 pathways.

ErbB2 Inhibition Leads to a Persistent ERK Phosphorylation By Testosterone

There is available data regarding a similar cross-talk between AR and ErbB2 in prostate cancer. A suggested mechanism for this cross-talk is ErbB2-mediated activation of AR through the ERK in prostate cancer [17,18]. In addition, ERK itself is a target of AR activation [17,19]. In view of these, we studied the role of the ERK pathway as a possible mechanism for the AR-ErbB2 cross-talk in MDA-MB-453 cells. The activation of ERK pathway was measured using ELISA (see Materials and Methods section). We observed that testosterone, as previously been reported [20], transiently increased the phosphorylation of ERK1/2 after a short incubation at 1 hour ($P < .01$), and this effect disappeared after a longer incubation at 18 hours (Figure 3A). However, when cells were treated with a combination of testosterone and ErbB2 inhibitor AG825, we found that the increase in ERK1/2-phosphorylation was not only present at 1 hour but also persisted after 18 hours of incubation ($P < .01$; Figure 3A). Similar results were obtained with testosterone treatment at 100 nM and 1 μ M concentrations. It has been shown that a physiologic response to testosterone involves a transient phosphorylation of ERK and persistent ERK phosphorylation, down-regulates cAMP response element-binding protein activity, leading to the inhibition of both cAMP response element-mediated gene expression and cell growth [20]. Furthermore, a novel inhibitor PM-20, which induces persistent phosphorylation of ERK1/2, has a growth-inhibitory effect on a number of cancer cell lines including hepatoma and breast cancer [21]. In view of these findings, we suggest a model for the AR-ErbB2 cross-talk in which ErbB2 activity is required for a physiologic transient phosphorylation of ERK1/2 by testosterone, and ErbB2 inhibition leads to a sustained testosterone-induced ERK1/2 phos-

phorylation resulting in the inhibition of downstream signaling and cell proliferation (Figure 3B). It is notable that we did not find a cross-talk at the phosphorylation steps of AR and ErbB2 (data not shown). However, it is still possible that the cross-talk exists at more than one level between AR and ErbB2 pathways.

The Cross-talk between AR and ErbB2 Affects Proliferation and Apoptosis

We next investigated the effect of cross-talk between AR and ErbB2 pathways on cell proliferation and apoptosis. Using MTT assay, we measured the proliferation of MDA-MB-453 and Sum-190 cells after treatments with testosterone, heregulin, AG825, and flutamide. Testosterone stimulated cell proliferation in both lines ($P < .01$; Figure 4, A and B), and this effect was blocked using anti-androgen flutamide and ErbB2 inhibitor AG825 (Figure 4, A and B). In addition, heregulin enhanced proliferation in MDA-MB-453 cells ($P < .01$), which was also reversed using AG825 and flutamide (Figure 4A). Furthermore, the combination of AG825 and flutamide showed a significant synergy against cell proliferation in both lines ($P < .01$; Figure 4, A and B). These findings suggest a therapeutic advantage in combined blockage of AR and ErbB2 pathways. We also investigated the effect of combined inhibition of AR and ErbB2 on apoptosis. Flutamide and AG825 were applied at different concentrations and apoptosis was assessed using Hoechst and Annexin V assays. We observed a significant additive effect in the proapoptotic response to flutamide at 40 μ M and AG825 at 20 μ M ($P < .03$; Figure 4, C and D). This effect was also present in the lower concentrations of these inhibitors (data not shown). These data suggest a cross-talk between AR and ErbB2 pathways affecting cell proliferation and apoptosis which has potential therapeutic implications in the molecular apocrine subtype of breast cancer. Interestingly, a similar observation in prostate cancer has revealed that simultaneous down-regulation of AR and ErbB2 pathways significantly impairs the survival of prostate cancer cells [12]. Future studies are needed to

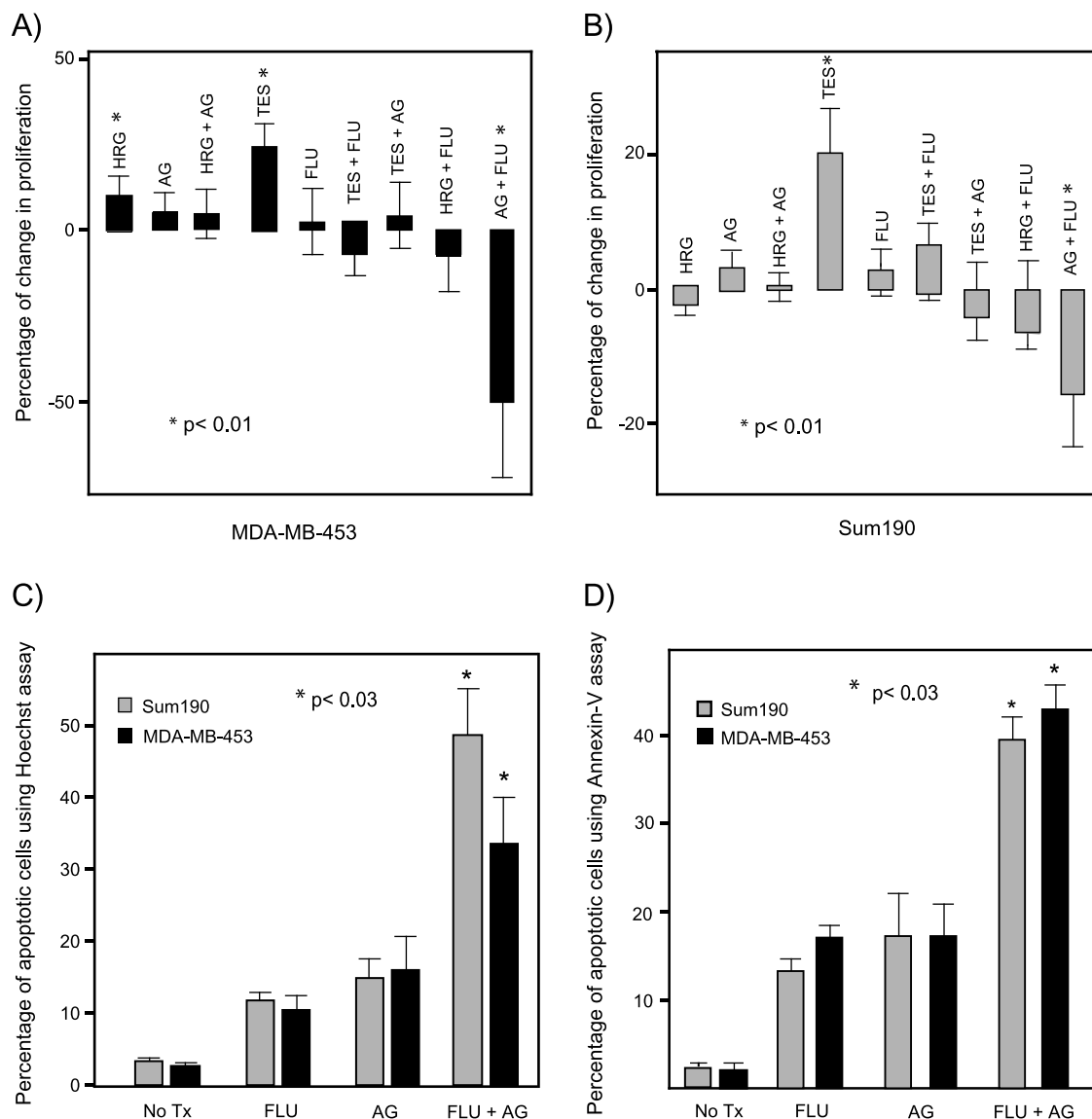


Figure 4. Synergy and additive effects with combined AR and ErbB2 inhibitions. (A) Proliferation assay in the MDA-MB-453 cell line using MTT. The percentage of change in proliferation is relative to the untreated cells. HRG indicates heregulin at 1 nM; AG, AG825 at 5 μ M; TES, testosterone at 1 μ M; FLU, flutamide at 10 μ M. * P < .01 is for each treatment group compared with untreated control using Mann-Whitney U test. AG + FLU combination is compared with AG and FLU groups separately (P < .01). Bars, SEM. (B) Proliferation assay in Sum-190 cell line performed as described in panel (A). (C) The percentage of apoptotic cells using Hoechst staining in MDA-MB-453 and Sum-190 cell lines. No Tx indicates no-treatment group; FLU, flutamide at 40 μ M; AG, AG825 at 20 μ M. * P < .03 is for FLU + AG group compared with the groups with FLU or AG treatment alone. (D) The percentage of apoptotic cells using Annexin V assay in MDA-MB-453 and Sum-190 cell lines. FLU indicates flutamide at 40 μ M; AG, AG825 at 20 μ M. * P < .03 is for FLU + AG group compared with the groups with FLU or AG treatment alone. All P values are calculated using Mann-Whitney U test.

examine the clinical implications of these findings in the management of molecular apocrine breast cancer.

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